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BASIC SCIENCE SHORT REPORT

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MiRNAs as biomarkers of phenotype in neutral lipid storage disease with myopathy

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Abstract

Background: Neutral lipid storage disease with myopathy (NLSDM) is a rare lipid metabolism disorder. In this study, we evaluated some circulating miRNAs levels in serum samples and the MRI of three affected siblings.

Methods: Three members of one NLSDM family were identified: two brothers and one sister. Muscles of lower and right upper extremities were studied by MRI. Expression profile of miRNAs, obtained from serum samples, was detected using qRT-PCR.

Results: Two brothers presented with progressive skeletal myopathy, while the sister had severe hepatosteatosis and diabetes. NLSDM patients showed a significant increase of muscle-specific miRNAs expression compared with healthy subjects. We found a correlation between hepatic damage and elevation of miRNAs expression profile of liver origin.

Conclusions: The dysregulation of miRNAs might represent an indicator of skeletal and hepatic damage and it might be useful to monitor the progression of NLSDM.

KEYWORDS

lipid metabolism, miRNAs, myomiRs, neutral lipid storage disease with myopathy, *PNPLA2*

1 | INTRODUCTION

Neutral lipid storage disease with myopathy (NLSDM) is a rare metabolic disorder, associated with mutations of the patatin like phospholipase domain containing 2 (*PNPLA2*) gene. *PNPLA2* encodes adipose triglyceride lipase, responsible for catalyzing triacylglycerol (TAG) breakdown.[1]

To date, only 56 patients have been described, mostly from Mediterranean countries; cases from Japan, China, and the United States have also been reported.[2,3] There is great variability in phenotype, but muscle weakness is usually the first symptom, resulting in both proximal and distal involvement.[3-5] Muscle biopsy reveals massive lipid storage, muscle atrophy, and rarely fiber necrosis. Heart and liver injury have been observed in nearly 40% and 20% of patients, respectively.[2,6,7] It is difficult to correlate the severity of clinical symptoms with *PNPLA2* mutations. Most gene variations lead to total loss or a severe decrease of lipase activity,[3-5] but in some patients these mutations are associated with late and/or mild progressive myopathy.[3,5] NLSDM pathophysiology is still largely unclear.

Abbreviations: CT, computed tomography scan; myomiRs, muscle-specific miRNA, microRNA; NLSDM, neutral lipid storage disease with myopathy; *PNPLA2*, phospholipase domain containing 2 gene; qRT-PCR, real-time polymerase chain reaction; SIRT1, Sirtuin 1; TAG, triacylglycerol.

Valentina Pegoraro and Sara Missaglia contributed equally to this study.

TABLE 1 Clinical and biochemical findings of neutral lipid storage disease with myopathy patients

		Patient 1	Patient 2	Patient 3
Age		61 years	58 years	50 years
Sex		M	F	M
Age of NLSDM onset		38 years	50 years	35 years
Neutral lipid accumulation	Granulocytes	severe	severe	severe
	Muscle	severe	ND	severe
Myopathy	Muscle weakness	severe	Mild weakness only in right triceps brachii muscle	Progressive in right upper extremity
	Muscle atrophy	severe	No	asymmetric distal atrophy
	CK (normal range: 0–180 IU/L)	804–1118	1098	1389
Cardiomyopathy		No	No	No
Liver	Hepatosteatosis	Mild hepatic steatosis	severe	No
	Hepatic enzymes (normal range: AST 4–40 U/L; ALT 4–40 U/L)	AST: 79 ALT: 72	AST: 127 ALT: 167	AST: 45 ALT: 57
Diabetes		No	Yes	No
Plasma triglycerides (normal range: 40–175 mg/dL)		139	125	233

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase.

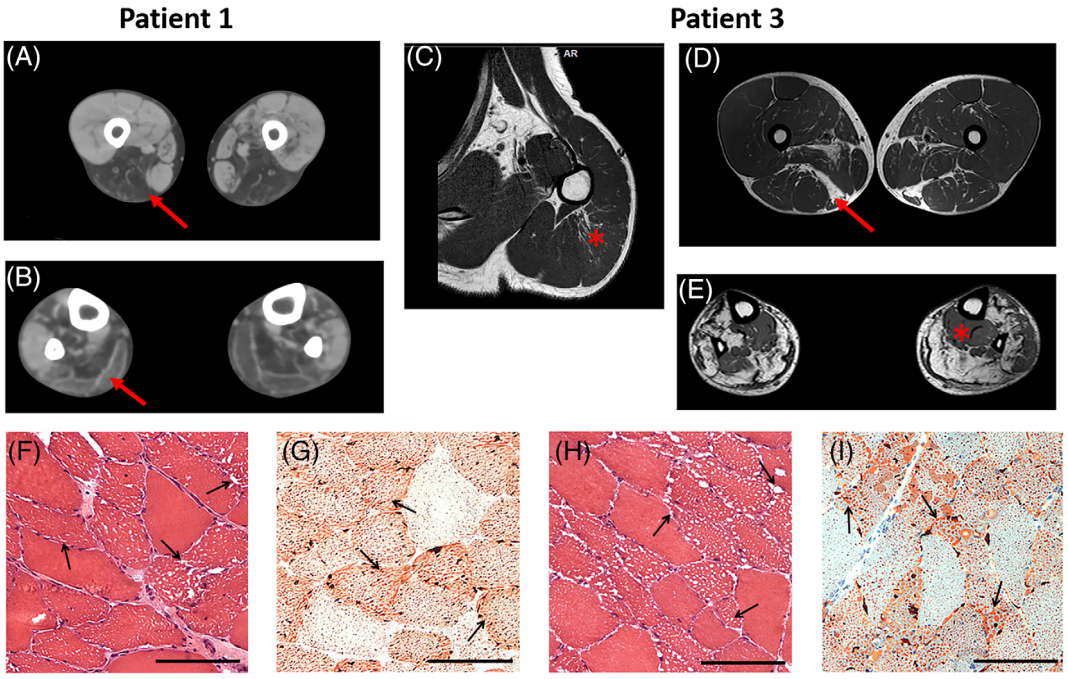

FIGURE 1 Muscle imaging and biopsy of patients 1 and 3. A, CT scan of patient 1 showed severe adipose tissue substitution in posterior thigh muscles, such as in semimembranosus and semitendinosus (arrow). B, Muscles of the posterior legs, mainly soleus and gastrocnemius, are completely substituted with fatty infiltration (arrow). MRIs of patient 3 revealed relative preservation of deltoid muscle (asterisk, C) and a slight fibro-fatty replacement of posterior thigh muscle such as adductor magnus and semimembranosus (arrow, D). E, In the posterior leg, fatty infiltration is more marked than in the anterior leg and only tibialis posterior and soleus muscles are spared (asterisk). Muscle biopsies showed vacuoles filled with abnormal lipid droplets (arrows) detected by hematoxylin and eosin stain in patient 1 (F) and patient 3 (H), and Oil-Red-O stain in patient 1 (G) and patient 3 (I) (scale bar = 100 μm). Abnormal lipid droplet accumulation is mostly observed in type I fibers [Color figure can be viewed at wileyonlinelibrary.com]

miRNAs are endogenous, small, single-strand, noncoding RNAs that act as critical regulators of gene expression at a posttranscriptional level.[8] Some miRNAs are tissue-specific, while others are expressed in multiple tissues. There is evidence that miRNAs are also present in plasma and serum, and, for this reason, are called circulating miRNAs. The function and origin of circulating miRNAs are not yet well known.[8,9] In particular,

muscle-specific miRNAs (myomiRs) may be secreted by skeletal muscle fibers into the systemic circulation actively or passively.[9]

Alterations of circulating miRNAs have been documented in several muscular dystrophies and other clinical conditions, such as obesity and insulin resistance. Therefore, they could be used as biomarkers of progression in neuromuscular and metabolic disorders.[10-13]

The myomiRs act as muscle regeneration and differentiation regulators and their balance aids in maintaining muscle homeostasis under normal cellular conditions.[14] MiR-206 plays a crucial role in muscle regeneration and maturation[15] and is widely expressed in newly-formed muscle fibers. It is also implicated in the modulation of muscle mass.[16] Both miR-1 and miR-206 promote myogenic differentiation, while miR-133a and miR-133b maintain the undifferentiated state and promote cell growth.[17] Moreover, in some muscle disorders, such as Duchenne muscular dystrophy and myotonic dystrophy type 1, it has been reported that increases of miR-206, miR-1, miR-133a, and miR-133b, may be useful biomarkers for monitoring muscle atrophy and evaluating treatment outcomes.[11,18]

Muscle imaging is another emerging diagnostic tool for detecting affected muscles through the identification of early fatty replacement and the presence of intramuscular edema. It may be useful for following disease progression.

In this study, we evaluated the levels of myomiRs and miRNAs involved in lipid metabolism in an Italian family, in which three members were affected. Moreover, we correlated miRNAs expression profile with muscle imaging results.

2 | METHODS

We enrolled four members of one NLSDM family (patient 1, the oldest brother [P1]; patient 2, the sister [P2]; patient 3, the younger brother [P3]; the daughter of P1, heterozygous carrier) and 7 healthy subjects (5 males and 2 females) matched for sex and age to patients.

The clinical data were collected during several outpatient examinations.

Muscle qualitative evaluation was performed by computed tomography (CT) scan and magnetic resonance imaging (MRI). In P3, images of lower limb and right upper extremity muscles were acquired using T1-weighted sequences by MRI. In P1, due to previous surgery, we could only perform CT scanning. Muscle pattern was ranked according to the Mercuri scale.[19] All procedures were approved by the local Institutional Review Board and conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. All patients signed a written, informed consent.

Histopathological evaluation of quadriceps muscles of P1 and P3 was previously performed for diagnostic reason.

The miRNAs were isolated from 400 μL of serum using the miRNeasy Mini Kit (QIAzol). A total of 10 ng of RNA were used to determine individual miRNA level by real-time polymerase chain reaction (qRT-PCR), performed using the CFX96™ Real-Time PCR detection System (Bio-Rad), with specific TaqMan MicroRNA Assay. The expression level of each miRNA was normalized to miR-16, U6 snRNA,

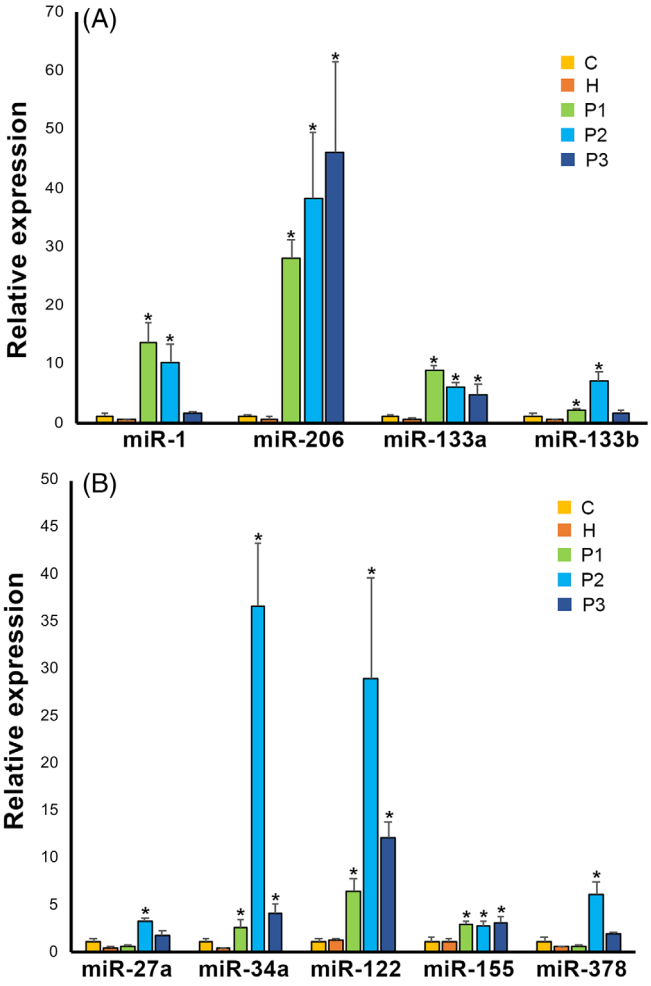

FIGURE 2 Dysregulation of circulating selected miRNAs in a NLSDM family in comparison to a control group. The bar graph represents the relative expression of serum myomiRs (A) and miRNAs involved in lipid metabolism (B) in NLSDM patients, compared with healthy subjects. Data are presented as mean + SD, with control value set to 1. *$P \leq .05$ (*). C, pool of controls; H, heterozygous carrier; P1, daughter; P1, NLSDM male patient; P2, NLSDM female patient; P3, NLSDM male patient [Color figure can be viewed at wileyonlinelibrary.com]

and miR-39-3p of *Caenorhabditis elegans*, the first two as internal controls and the other as spike-in miRNA, respectively. Primers sequences are in Supporting Information Table S1, which is available online. Data were obtained from at least three qRT-PCR experiments and each sample was analyzed in duplicate. Relative expression was calculated using the comparative ΔΔCt method. The Wilcoxon-Mann–Whitney test for paired data for small samples was used to asses significance. The level of statistical significance was set at $P \leq .05$.

3 | RESULTS

We studied three affected family members, carrying two heterozygous compound *PNPLA2* mutations.[6] These subjects exhibited highly variable clinical features. Two male siblings, aged 61 and 50 years, had

an asymmetric distal myopathy, while the 58-year-old sister showed hepatosteatosis and type 2 diabetes as the main findings. Clinical and laboratory findings are summarized in Table 1. CT scan (P1) and MRI (P3) revealed fibro-fatty replacement in the lower and right upper extremities (Figure 1A-E); lipid droplets were observed in muscle biopsy of both patients (Figure 1F-I).

Expression level analysis revealed that patients exhibited a significant elevation of serum myomiRs (Figure 2A). In particular, miR-206 was 28- to 38-fold higher than in control subjects, while miR-133a was 4.7- to 9-fold higher. Also, miR-1 was increased in P1 and P2 up to 10-fold, and miR-133b approximately 2-fold.

The investigation of some circulating miRNAs, mainly involved in lipid metabolism, showed a significant up-regulation of miR-122, miR-34a, and miR-155 ($P < .05$) in NLSDM patients, compared with control group (Figure 2B). The expression profile of miR-378 and miR-27a was significantly elevated only in P2 (Figure 2B).

4 | DISCUSSION

In our NLSDM brothers, CT scan/muscle MRI demonstrated that muscle mass correlated with miR-206 levels. P1 presented walking difficulties and advanced myopathy, whereas P3 had a slowly evolving myopathy. Because miR-206 expression was lower in P1 than in P3, it could be hypothesized that severe muscle damage partially or totally prevents muscle regeneration.

In P2, who was minimally symptomatic, hepatosteatosis was detected by ECHO and a significant overexpression of miR-1 and miR-206 was found. This can be explained by the observation that miR-1 and miR-206 are involved in lipid accumulation in hepatocytes.[20]

The miRNAs can also play an important role in the regulation of lipid metabolism. The first identified was miR-122. Its suppression causes a reduction of circulating cholesterol and TAG levels as well as of hepatosteatosis.[21] miR-34a regulates Sirtuin 1 (SIRT1) expression and, consequently, plays important role in lipid metabolism.[22] In pathological conditions, such as nonalcoholic fatty liver disease and obesity, the overexpression of miR-34a causes SIRT1 inhibition, inducing hepatic inflammation, a decrease of lipid metabolism gene expression and increase of de novo lipogenesis.[13,23] In our patients, we found a positive correlation between the increase of miR-122 and miR-34a and high levels of hepatic enzymes. In particular, we observed an increase of these miRNAs in P2, who had hepatosteatosis, diabetes, and abdominal fat accumulation.

It has been reported that miR-378 induces the expression of adipocyte genes and lipid storage during adipogenesis,[24] while miR-27a negatively regulates the expression of some lipid metabolic genes.[20] Consistent with this, these miRNAs showed a significant increase only in P2. Recently, some studies showed that miR-378 is also expressed in skeletal muscle and an increase of its plasma level was reported in animal models of muscular dystrophy,[25,26] and acute muscle injury.[27] These data are not in agreement with the miR-378 profile observed in NLSDM patients, as this miRNA was not significantly upregulated in those patients showing substantial muscle involvement (P1, P3). Further studies are needed to clarify whether miR-378 and miR-27a play a role in human muscle metabolic disorders.[28]

Finally, our results also showed an increase of miR-155 in NLSDM patients in comparison to the control group. MiR-155 has important functions in inflammation and, recently, was described to contribute to liver pathology and arteriosclerosis.[29,30]

In conclusion, we show that the increased expression levels of muscle and lipid metabolism-specific miRNAs may be an indicator of clinical phenotype. Because of the tissue-specific distribution of numerous miRNAs, they are excellent biomarkers of muscle, heart, or liver injuries. In NLSDM, multiple organs are affected; therefore, this disease could have a very special serum miRNA signature providing a powerful new tool. This could be further studied with more complete profiling.

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ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

REFERENCES

1. Missaglia S, Coleman RA, Mordente A, Tavian D. Neutral lipid storage diseases as cellular model to study lipid droplet function. *Cells*. 2019; pii: E187.
2. Higashi M, Hirano K, Kobayashi K, et al. Distinct cardiac phenotype between two homozygotes born in a village with accumulation of a genetic deficiency of adipose triglyceride lipase. *Int J Cardiol*. 2015; 192:30-32.
3. Pennisi EM, Arca M, Bertini ES, et al. Neutral lipid storage diseases: clinical/genetic features and natural history in a large cohort of Italian patients. *Orphanet J Rare Dis*. 2017;90:1-10.
4. Tavian D, Missaglia S, Redaelli C, et al. Contribution of novel ATGL missense mutations to the clinical phenotype of NLSD-M: a strikingly low amount of lipase activity may preserve cardiac function. *Hum Mol Genet*. 2012;21:5318-5328.
5. Missaglia S, Maggi L, Mora M, et al. Late onset of neutral lipid storage disease due to novel PNPLA2 mutations causing a total loss of lipase activity in a patient with myopathy and slight cardiac involvement. *Neuromuscul Disord*. 2017;27:481-486.
6. Missaglia S, Tasca E, Angelini C, Moro L, Tavian D. Novel missense mutations in PNPLA2 causing late onset and clinical heterogeneity of neutral lipid storage disease with myopathy in three siblings. *Mol Genet Metab*. 2015;115:110-117.
7. Pasanisi MB, Missaglia S, Cassandrini D, et al. Severe cardiomyopathy in a young patient with complete deficiency of adipose triglyceride lipase due to a novel mutation in PNPLA2 gene. *Int J Cardiol*. 2016; 207:165-167.
8. Bhaskaran M, Mohan M. MicroRNAs: history, biogenesis, and their evolving role in animal development and disease. *Vet Pathol*. 2014;51: 759-774.

9. Siracusa J, Koulmann N, Banzet S. Circulating myomiRs: a new class of biomarkers to monitor skeletal muscle in physiology and medicine. *J Cachexia Sarcopenia Muscle*. 2018;9:20-27.
10. Tasca E, Pegoraro V, Merico A, Angelini C. Circulating microRNAs as biomarkers of muscle differentiation and atrophy in ALS. *Clin Neuropathol*. 2016;35:22-30.
11. Cacchiarelli D, Legnini I, Martone J, et al. miRNAs as serum biomarkers for Duchenne muscular dystrophy. *EMBO Mol Med*. 2011;3:258-265.
12. Matsuzaka Y, Kishi S, Aoki Y, et al. Three novel serum biomarkers, miR-1, miR-133a, and miR-206 for Limb-girdle muscular dystrophy, facioscapulohumeral muscular dystrophy, and Becker muscular dystrophy. *Environ Health Prev Med*. 2014;19:452-458.
13. Suksangrat T, Phannasil P, Jitrapakdee S. miRNA regulation of glucose and lipid metabolism in relation to diabetes and non-alcoholic fatty liver disease. In: Guest P, ed. *Reviews on Biomarker Studies of Metabolic and Metabolism-Related Disorders. Advances in Experimental Medicine and Biology*. Berlin: Springer, Cham; 2019. vol 1134.
14. Mitchelson KR, Qin WY. Roles of the canonical myomiRs miR-1, −133 and −206 in cell development and disease. *World J Biol Chem*. 2015;6:162-208.
15. Horak M, Novak J, Bienertova-Vasku J. Muscle-specific microRNAs in skeletal muscle development. *Dev Biol*. 2016;410:1-13.
16. Ma G, Wang Y, Li Y, et al. MiR-206, a key modulator of skeletal muscle development and disease. *Int J Biol Sci*. 2015;11:345-352.
17. Townley-Tilson WH, Callis TE, Wang D. MicroRNAs 1, 133, and 206: critical factors of skeletal and cardiac muscle development, function, and disease. *Int J Biochem Cell Biol*. 2010;42:1252-1255.
18. Koutsoulidou A, Kyriakides TC, Papadimas GK, et al. Elevated muscle-specific miRNAs in serum of myotonic dystrophy patients relate to muscle disease progress. *PLoS One*. 2015;10:e0125341.
19. Mercuri E, Pichiecchio A, Counsell S, et al. A short protocol for muscle MRI in children with muscular dystrophies. *Eur J Paediatr Neurol*. 2002;6:305-307.
20. Zhong D, Huang G, Zhang Y, et al. MicroRNA-1 and microRNA-206 suppress LXRα-induced lipogenesis in hepatocytes. *Cell Signal*. 2013;25:1429-1437.
21. Esau C, Davis S, Murray SF, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab*. 2006;3:87-98.
22. Lee J, Padhye A, Sharma A, et al. A pathway involving farnesoid X receptor and small heterodimer partner positively regulates hepatic sirtuin 1 levels via microRNA-34a inhibition. *J Biol Chem*. 2010;285:12604-12611.
23. Fu T, Seok S, Choi S, et al. microRNA 34a inhibits beige and brown fat formation in obesity in part by suppressing adipocyte fibroblast growth factor 21 signaling and SIRT1 function. *Mol Cell Biol*. 2014;34:4130-4142.
24. Krist B, Florczyk U, Pietraszek-Gremplewicz K, Józkowicz A, Dulak J. The role of miR-378a in metabolism, angiogenesis, and muscle biology. *Int J Endocrinol*. 2015;2015:281756.
25. Vignier N, Amor F, Fogel P, et al. Distinctive serum miRNA profile in mouse models of striated muscular pathologies. *PLoS One*. 2013;8:e55281.
26. Jeanson-Leh L, Lameth J, Krimi S, et al. Serum profiling identifies novel muscle miRNA and cardiomyopathy related miRNA biomarkers in Golden Retriever muscular dystrophy dogs and Duchenne muscular dystrophy patients. *Am J Pathol*. 2014;184:2885-2898.
27. Siracusa J, Koulmann N, Bourdon S, Goriot ME, Banzet S. Circulating miRNAs as biomarkers of acute muscle damage in rats. *Am J Pathol*. 2016;186:1313-1327.
28. Chemello F, Grespi F, Zulian A, et al. Transcriptomic analysis of single isolated myofibers identifies miR-27a-3p and miR-142-3p as regulators of metabolism in skeletal muscle. *Cell Rep*. 2019;26:3784-3797.
29. Pergande MR, Cougnoux A, Rathnayake RAC, Porter FD, Cologna SM. Differential proteomics reveals miR-155 as a novel indicator of liver and spleen pathology in the symptomatic Niemann-Pick Disease, type C1 mouse model. *Molecules*. 2019;24:994.
30. Bruen R, Fitzsimons S, Belton O. miR-155 in the resolution of atherosclerosis. *Front Pharmacol*. 2019;10:463.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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